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(54) Title: PATHOGEN ASSAY METHOD

(57) Abstract

A method for detecting a pathogen in a sample comprises the steps of: (i) optionally treating the sample to obtain a pathogen-enriched sample, (ii) mixing a predetermined amount of the optionally pathogen-enriched sample with a solution containing a predetermined amount of antibody capable of specifically binding to the pathogen to permit the antibody to bind to the pathogen, (iii) separating any pathogen from the mixture to obtain a pathogen-free solution, and (iv) determining the amount of antibody present in the pathogen-free solution to thereby detect binding of antibody to the pathogen as indicative of the presence of pathogen in the sample.

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PATHOGEN ASSAY METHOD

The present invention relates to a novel assay method for detecting pathogens, especially in foods.

The statistics of food poisoning incidences in recent years, the strict food safety legislation and introduction of quality systems in food manufacturing has increased the need for real-time techniques for pathogen analysis during food production.

The conventional cultural method for the detection of pathogens like *Salmonella* and *Listeria* requires at least five days to isolate and identify the organism from a food product. In foods, *Salmonella* and *Listeria* are often present in low numbers, largely outnumbered by competing organisms, and occasionally in a stressed state. The method therefore includes an initial non-selective pre-enrichment stage designed to allow the resuscitation and growth of the target pathogen. The subsequent selective enrichment stage utilises growth media that are inhibitory to competitor cells whilst still maintaining or allowing the growth of the target organism. The selective enrichment process aims to improve the ratio of *Salmonella* or *Listeria* to competitor cells. Colonies are then isolated from the enriched culture by plating on to selective and differential agar media. Any presumptive-positive colony is then confirmed biochemically and identified serologically. While cultural techniques for the microbiological examination of foods have high sensitivity (capable of detecting one viable cell), they are time-consuming and highly labour- and material-intensive.

Considerable research has therefore been focused on the development of rapid methods for the detection of pathogens in foods. However, none of the rapid methods available commercially so far, such as ELISA, impedance, and DNA hybridisation, give results in real-time but all require at least 24 to 48 h of cultural enrichment in order to increase the productivity of the pathogens above the

detection limits of these techniques, which at present is above 10^5 cfu/ml.

The available immunoassays of the enzyme-linked immunosorbent assay (ELISA) type for *Salmonella*, for example, are to be performed on selectively enriched samples (48-h culture) and comprise heat-solubilising *Salmonella* antigen, adding the antigen-containing broth to antibody-coated wells of a microtitre plate and incubating, adding antibody-enzyme conjugate, and after incubation adding substrate and reading absorbance.

The object of the present invention is to provide a pathogen assay which together with a rapid assay protocol has a sufficiently low detection limit, such as 10^2-10^4 cfu/ml, to permit detection of pathogens in contaminated foods without 24-h selective enrichment of the food sample, and therefore within a single working day (less than 24 h).

In accordance with the present invention it has now been found that this object may be achieved by a modified inhibition type immunoassay wherein a possibly pathogen-containing sample suspension is first reacted with a predetermined excess level of antibodies against the pathogen. The suspension is then subjected to a separation procedure to remove the pathogen, and the resulting antibody level in the separated solution is determined to thereby indirectly detect the pathogen. A detected low relative level of antibody will indicate a high pathogen level in the reacted sample, whereas a detected high level of antibody will indicate a low pathogen level in the sample.

Accordingly, the present invention in a broad aspect provides a method for detecting a pathogen in a sample, comprising the steps of:

(i) optionally treating the sample to obtain a pathogen-enriched sample (in case of pathogen in the sample),

(ii) mixing a predetermined amount of the optionally pathogen-enriched sample with a solution containing a predetermined amount of an antibody capable of specifically

binding to the pathogen to permit the antibody to bind to the pathogen,

(iii) separating any pathogen from the mixture to obtain a pathogen-free solution, and

(iv) determining the amount of antibody present in the pathogen-free solution to thereby detect binding of antibody to the pathogen as indicative of the presence of pathogen in the sample.

The antibody is preferably added in excess of the amount corresponding to the (expected) maximum amount of pathogen to leave unbound antibody in the reaction mixture.

The term pathogen as used herein comprises not only bacteria, but also virus, fungi and protozoa.

Although the detection of foodborne pathogens is an important application of the present invention, the method may, of course, also be applied to samples of other origin, such as, e.g., samples for clinical assays, e.g., blood, urine, etc.

Exemplary of common foodborne pathogens are *Salmonella* and *Listeria*. Adequate pre-enrichment (and optionally 6-h selective enrichment) procedures for food samples to reach a pathogen level of about 10^2-10^4 cfu/ml as mentioned above are well-known to those skilled in the art.

The removal of the pathogen from the antibody reaction mixture is preferably performed by filtration or centrifugation. Suitable filters for this purpose are known to a person skilled in the art and will not be described in detail herein.

The technique chosen for detecting the excess antibody in the pathogen-free solution is not critical per se. In a preferred detection technique, however, the solution is contacted with an optical sensor surface having immobilized thereon a receptor, such as an antibody, for the primary antibody, and the change in refractive index at the surface related to binding of primary antibody present in the solution to the sensor surface is measured.

Advantageously, the contacting of the sample with the surface is performed by passing the sample over the surface utilizing a liquid flow system, i.e. a flow cell.

If desired, further sensitivity in the assay may be obtained by using a secondary reagent capable of binding to the primary antibody bound to the sensor surface. Optionally, also a tertiary reagent may be used which binds to the secondary reagent.

Binding of the receptor, such as an antibody, to the surface may be carried out in conventional ways well-known to those skilled in the art. If, for example, the optical surface has a polymeric organic layer at its surface, the receptor may be directly covalently bound to the surface using known linker reagents. Alternatively, an intermediate ligand, such as an antibody, which binds the receptor may first be covalently bound to the surface before this bound intermediate ligand is exposed to the receptor for the analyte to bind this to the surface. It will be appreciated that covalent and/or affinity bonding may be effective in binding the receptor to the surface. However, when the receptor is bound to the surface, it is important that its ability to bind the ligand, i.e. in this case the primary antibody, should remain unchanged.

The term antibody as used herein is to be interpreted broadly. Thus, in addition to a whole antibody, the antibody may be a fragment thereof, such as an Fab fragment, an Fv fragment, a single chain fragment (scFv), a single heavy chain or even a peptide (based on the nucleotide sequence of the antibody gene) having binding activity. The antibodies which may be used in the invention may be obtained by conventional methods and are many times commercially available. Although polyclonal antibodies may conveniently be used in the method of the invention, monoclonal antibodies may be preferred at least in certain cases for their greater specificity.

The measurement of the change in refractive index at the surface may advantageously be based on evanescent wave sensing, such as surface plasmon resonance spectroscopy

(SPRS), Brewster angle refractometry, critical angle refractometry, frustrated total reflection (FTR), evanescent wave ellipsometry, scattered total internal reflection (STIR), optical wave guide sensors, evanescent wave based imaging, such as critical angle resolved imaging, Brewster angle resolved imaging, SPR angle resolved imaging, etc. In the currently preferred method for carrying out the invention, the measurement is based on surface plasmon resonance. This technique is described, inter alia, in EP-A-0305109, EP-A-0267142 and WO-A-90/05295. The optical surface which is used in the measurement based on surface plasmon resonance preferably comprises a gold film and a hydrogel bound to the gold film, as described in WO 90/05303. This type of optical surface may easily be regenerated so that a single surface may be used for many analyses. The overall cost per analysis can therefore be reduced considerably. Suitable apparatus incorporating such an optical surface is the BIAcore® system available from Pharmacia Biosensor AB, (Uppsala, Sweden) the methods of operation of which are described in the BIAcore® Methods Manual (Pharmacia Biosensor AB). In the BIAcore® system, a flow system passes the sample over a replaceable sensor chip forming one wall of a flow cell. The sensor chip supports a gold layer which typically has a thickness of 50 nm. A carboxylated dextran is bound to the gold layer via a linker layer. To this dextran layer the receptor for the primary antibody may be bound.

The invention will now be described in more detail with reference to the following Examples and the accompanying drawings, wherein:

Fig. 1 is a diagram showing the dose-response curve for *S. enteritidis* and the response of *C. freundii* obtained by the method of the invention in the same biosensor flow cell;

Fig. 2 is diagram showing the dose-response curve for *S. typhimurium* and the response of *C. freundii* obtained by

the method of the invention in the same biosensor flow cell;

Fig. 3 is a diagram showing the dose-response curve for *S. napoli* and the response of 5 non-salmonellae obtained by the method of the invention in the same biosensor flow cell;

Fig. 4 is a diagram showing the dose-response curve for *S. stanley* and the response of 3 non-salmonellae obtained by the method of the invention in the same biosensor flow cell;

Fig. 5 is a diagram showing the dose-response curve for *S. thompson* and the response of 4 non-salmonellae obtained by the method of the invention in the same biosensor flow cell;

Fig. 6 is a diagram showing the dose-response curve for *S. typhimurium* and the response of *S. enteritidis, C. freundii* and *E. coli* obtained by the method of the invention in the same biosensor flow cell and using a monoclonal as primary antibody;

Fig. 7 is a diagram showing the dose-response curves for three *Listeria* serotypes and the response of three non-listeriae obtained by the method of the invention; and

Fig. 8 is a diagram showing the dose-response curves for five *Salmonella* serotypes and the response of 10 non-salmonellae obtained using a commercial prior art *Salmonella* ELISA (Salmonella-Tek; Organon).

The analyses in the Examples were carried out on a BIAcore® system (Pharmacia Biosensor AB, Uppsala, Sweden) with a Sensor Chip CM5 as the optical sensor surface.

EXAMPLES

MATERIALS AND METHODS

Buffers and regeneration solutions

Hepes buffered saline (HBS, Pharmacia Biosensor AB), containing 10 mM Hepes, pH 7.4, 150 mM sodium chloride (NaCl), 3.4 mM ethylenediaminetetraacetic acid (EDTA), and 0.005% v/v Surfactant P20, was used as the standard running buffer in all experiments.

Sodium acetate buffer (10 mM, pH 5) was used as the coupling buffer to enable immobilisation of the antibodies to the sensor surface.

The following solutions were tested for their ability to regenerate the sensor surface: formic acid (1M), hydrochloric acid (10-100 mM HCl), glycine/HCl (10 mM glycine titrated with HCl to required pH), ethanolamine/HCl (1M ethanolamine titrated with HCl to required pH), potassium hydroxide (10-100 mM KOH), potassium chloride (4 M KCl), sodium hydroxide (10-100 mM NaOH), KCl/NaOH (4 M KCl titrated with NaOH to required pH), glycine/NaOH (10 mM glycine titrated with NaOH to required pH), ethanolamine/NaOH (1 M ethanolamine titrated with NaOH to required pH), and sodium dodecyl sulphate (0.05-1 % SDS).

<u>Antibodies</u>

Bactrace anti-*Salmonella* CSA-1 (Kirkegaard & Perry Laboratories), below referred to as "KPL Bactrace"

Mouse anti-*Salmonella* broad specificity Mab (Serotec)

Bactrace anti-*Listeria* (genus specific, Dynatech)

Rabbit anti-goat IgG_{fab} ($RAGF_{ab}$, Pierce)

Goat anti-mouse IgG_{fab} ($GAMF_{ab}$, Pierce)

<u>Immobilisation of antibodies to the sensor surface</u>

Pharmacia Biosensor's recommended protocol was used for this purpose and involved the reagents (NHS, EDC and ethanolamine) contained in the amine coupling kit (code no. BR-1000-50, Pharmacia Biosensor AB).

The carboxylated dextran surface was initially activated using a 35 µl (flow rate of 5 µl/min) injection of N-hydroxysuccinimide (NHS) and N-ethyl-N^1(dimethylaminepropyl)carbodiimide (EDC) in distilled water (0.05 M NHS/0.2 M EDC), across a flow cell surface. This was then followed by an injection (35 µl) of the antibody in 10 mM sodium acetate buffer, pH 5, and a subsequent injection (35 µl) of 1 M ethanolamine hydrochloride.

<u>Microorganisms</u>

The following organisms were used: *Salmonella enteritidis* P167808, *S. typhimurium* NCTC 74, *S. napoli* NCTC

6853, *S. stanley* R23, *S. thompson* R24, *Listeria innocua* GP100/100, *L. monocytogenes* NCTC 5105, *L. innocua* LFRA isolate B047, *L. seeligeri* LFRA B052, *Bacillus cereus* NCTC 11145, *Staphylococcus aureus* NCTC 4136, *Staph. aureus* LFRA isolate B3, *Staph. epidermidis* LFRA isolate PB14, *Yersinia enterocolitica* NCTC 11174, *Escherichia coli* NCTC 9001, *E. coli* NCTC 9112, *E. coli* NCTC 8545, *Proteus mirabilis* 1102/11/1, *Citrobacter freundii* NCTC 6266 and *Micrococcus luteus* NCTC 7495.

All the cultures were grown at 37°C for 24 h in Trypticase Soya Broth (TSB). The cultures were then diluted (serial decimal) in HBS and enumerated by plating 0.1 ml on to Trypticase Soya Agar (TSA).

Regeneration of the sensor surface

The regeneration conditions were optimised for each anti-*Salmonella* and anti-*Listeria* antibody tested. This was achieved by screening a range of potential regeneration solutions, as specified in the BIAcore™ user manual, in order to find the best possible solution that completely dissociated the antibody from the sensor surface. Thus, the capture antibody, e.g. $RAGF_{ab}$, was immobilised to the sensor surface and the anti-*Salmonella* antibody, e.g. KPL Bactrace, was injected using the manual command mode to allow binding to the biosensor surface. Then a weak regeneration solution was injected for 1 min and the degree of regeneration monitored. It was found that for KPL Bactrace, for example, the best regeneration solution was 50 mM NaOH.

EXAMPLE 1: Inhibition assay for *Salmonella*

Determination of minimum relative response

Varying levels of an anti-*Salmonella* antibody were reacted with a high level (approximately 10^8 cfu/ml) of a *Salmonella* culture in order to determine the level of primary antibody required to give a minimum relative response in the BIAcore®.

Preparation of bacterial cultures

Bacterial cultures were grown at 37°C for 24 h in trypticase soya broth (TSB). The 24-h cultures were then

heated in boiling water for 20 min. Prior to assay, the heat-inactivated cultures were diluted in Hepes Buffered Saline (HBS).

Optical biosensor assay

500 µl of diluted primary antibody to *Salmonella*, corresponding to the above predetermined level, were pre-reacted with 500 µl of heat-inactivated bacterial cells and mixed for 15 min on a shaker (Luckham multimix). The mixture was then filtered with a 0.22 µm Sartorius filter to remove excess primary antibody from antibody bound to the bacterial cells, and 15 µl of the filtrate containing the separated excess primary antibody were injected over the sensor surface coated with the corresponding capture antibody. The sensor surface was then regenerated. The time to first result was 30 min. The higher the level of *Salmonella*, the lower was the relative response, and vice versa. The results are shown in Figs. 1 to 6.

Figs. 1 to 5 show obtained response curves for *S. enteritidis*, *S. typhimurium*, *S. napoli*, *S. stanley* and *S. thompson*, respectively, together with the response of various non-salmonellae challenged at approximately 10^8 cfu/ml in the same flow cell. In this case, a polyclonal antibody, KPL Bactrace, was used as primary antibody. Rabbit anti-goat IgG_{fab} fragment ($RAGF_{ab}$) was used as capture antibody on the sensor surface, and regeneration was performed with 50 mM sodium hydroxide, 2x1 min pulses. As appears from the Figs. 1-5, the detection limit for all five *Salmonella* serotypes was in the range 10^2-10^4 cfu/ml.

Fig. 6 shows the detection of *Salmonella* using a monoclonal anti-*Salmonella* (Serotec) as primary antibody and goat anti-mouse IgG_{fab} fragment ($GAMF_{ab}$) as capture antibody on the sensor surface. Regeneration of the $GAMF_{ab}$ sensor surface reacted with the primary antibody was performed using a combination of 1x15 µl formic acid (1M), 1x5 µl NaOH (2.5 mM) and 1x15 µl formic acid (1M) pulses. It is seen from the dose-response curve for *S. typhimurium* shown in Fig. 6 that no cross-reaction was observed for *C. freundii* and *E. coli* tested at approximately 10^8 cfu/ml. A

S. enteritidis control at approximately 10^8 cfu/ml was also successfully detected.

EXAMPLE 2: Inhibition assay for *Listeria*

In the same way as described for *Salmonella* above, an inhibition assay for three *Listeria* strains was performed using a $RAGF_{ab}$-coated sensor surface and a polyclonal anti-*Listeria* as primary antibody. The results are shown in Fig. 7. As appears therefrom, the sensitivity level of the assay was approximately 10^2-10^4 cfu/ml, with no cross-reaction observed for *E. coli*, *Staph. aureus* and *Staph. epidermidis* tested at approximately 10^8 cfu/ml.

EXAMPLE 3: Commercial *Salmonella* ELISA (comparative)

Bacterial cultures were prepared as in Example 1 above, and five *Salmonella* serotypes and responses of ten non-*Salmonella* were detected by a commercial *Salmonella* assay, "Salmonella-Tek ELISA", using the following protocol.

100 µl samples were pipetted into wells of a microtitre plate. The samples were then incubated at 37°C for 30 min. After washing six times with wash solution, 100 µl conjugate were added and incubated at 37°C for 30 min. The wells were then washed six times, and 100 µl of substrate were added and incubated at room temperature for 30 min. 100 µl of stop solution were then added and the absorbance was read at 450 nm. The time to first result was 2 h.

The results are shown in Fig. 8, demonstrating a detection limit of approximately 10^4-10^6 cfu/ml for salmonellae, with considerable cross-reaction from *C. freundii* and *E. coli* R6, but not eight of the other non-salmonellae all tested at approximately 10^8 cfu/ml.

CLAIMS

1. A method for detecting a pathogen in a sample, comprising the steps of:

(i) optionally treating the sample to obtain a pathogen-enriched sample,

(ii) mixing a predetermined amount of the optionally pathogen-enriched sample with a solution containing a predetermined amount of antibody capable of specifically binding to the pathogen to permit the antibody to bind to the pathogen,

(iii) separating any pathogen from the mixture to obtain a pathogen-free solution, and

(iv) determining the amount of antibody present in the pathogen-free solution to thereby detect binding of antibody to the pathogen as indicative of the presence of pathogen in the sample.

2. The method according to claim 1, wherein the antibody is added in step (ii) in a sufficient amount to leave unbound antibody in the mixture.

3. The method according to claim 1 or 2, wherein the determination of antibody in step (iv) comprises contacting at least a predetermined portion of the pathogen-free solution with an optical sensor surface having immobilized thereon a receptor for the antibody, and measuring a change in refractive index at the surface related to the amount of antibody present in the pathogen-free solution contacted with the sensor surface.

4. The method according to claim 1, 2 or 3, wherein the separation of the pathogen in step (iii) is performed by filtration.

5. The method according to any one of claims 1 to 4, wherein said receptor is an antibody.

6. The method according to any one of claims 1 to 5, wherein the pathogen is a foodborne pathogen and step (i) comprises enriching the pathogen in the food sample to a level in the range of from about 10^2 to about 10^4 cfu/ml.

7. The method according to any one of claims 1 to 6, wherein said measurement of the change of refractive index is based on internal reflection.

8. The method according to claim 7, wherein said measurement of the change of refractive index is based on surface plasmon resonance.

9. The method according to claim 8, wherein said optical sensor comprises a gold film and a hydrogel bound to the gold film.

10. The method according to any one of claims 1 to 9, wherein the sample is contacted with the optical sensor surface by passing the sample over the sensor surface in a liquid flow.

11. The method according to any one of claims 1 to 10, wherein the pathogen is selected from *Salmonella* and *Listeria*.

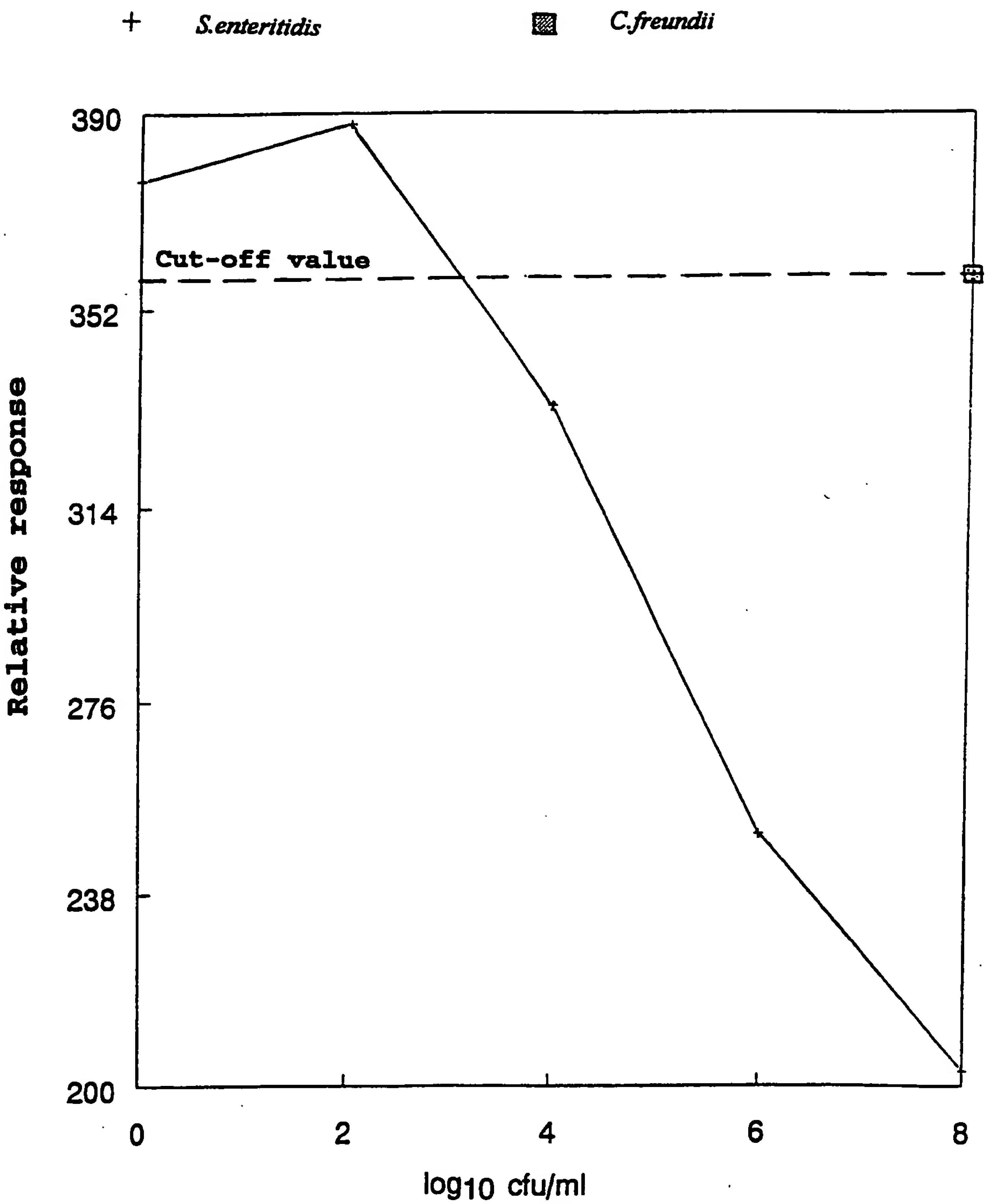
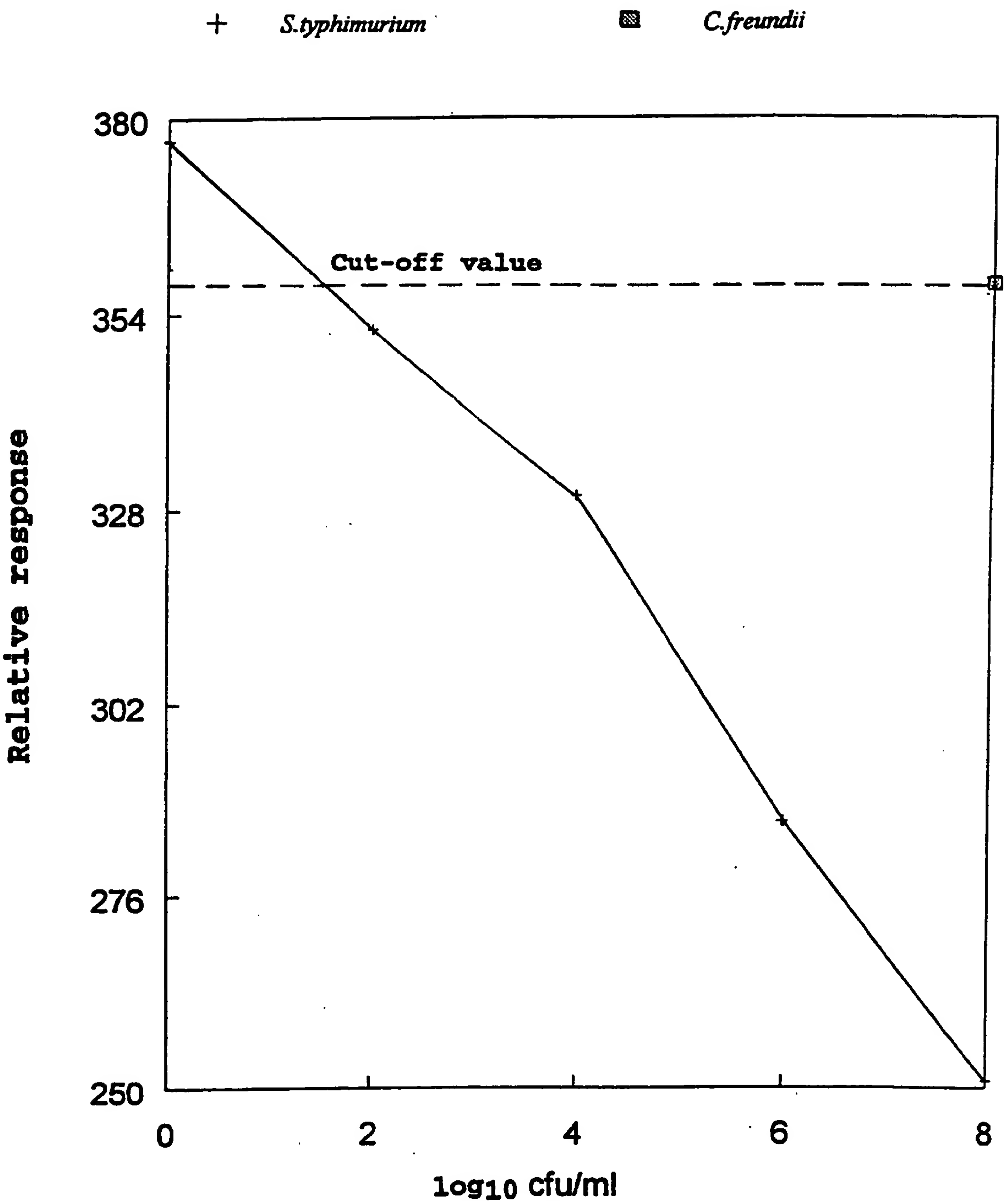

+ S.enteritidis
C.freundii
390
352
314
276
238
200
Cut-off value
Relative response
0
2
4
6
8
log10 cfu/ml

FIG. 1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C.freundii
Cut-off value
Relative response
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302
276
250
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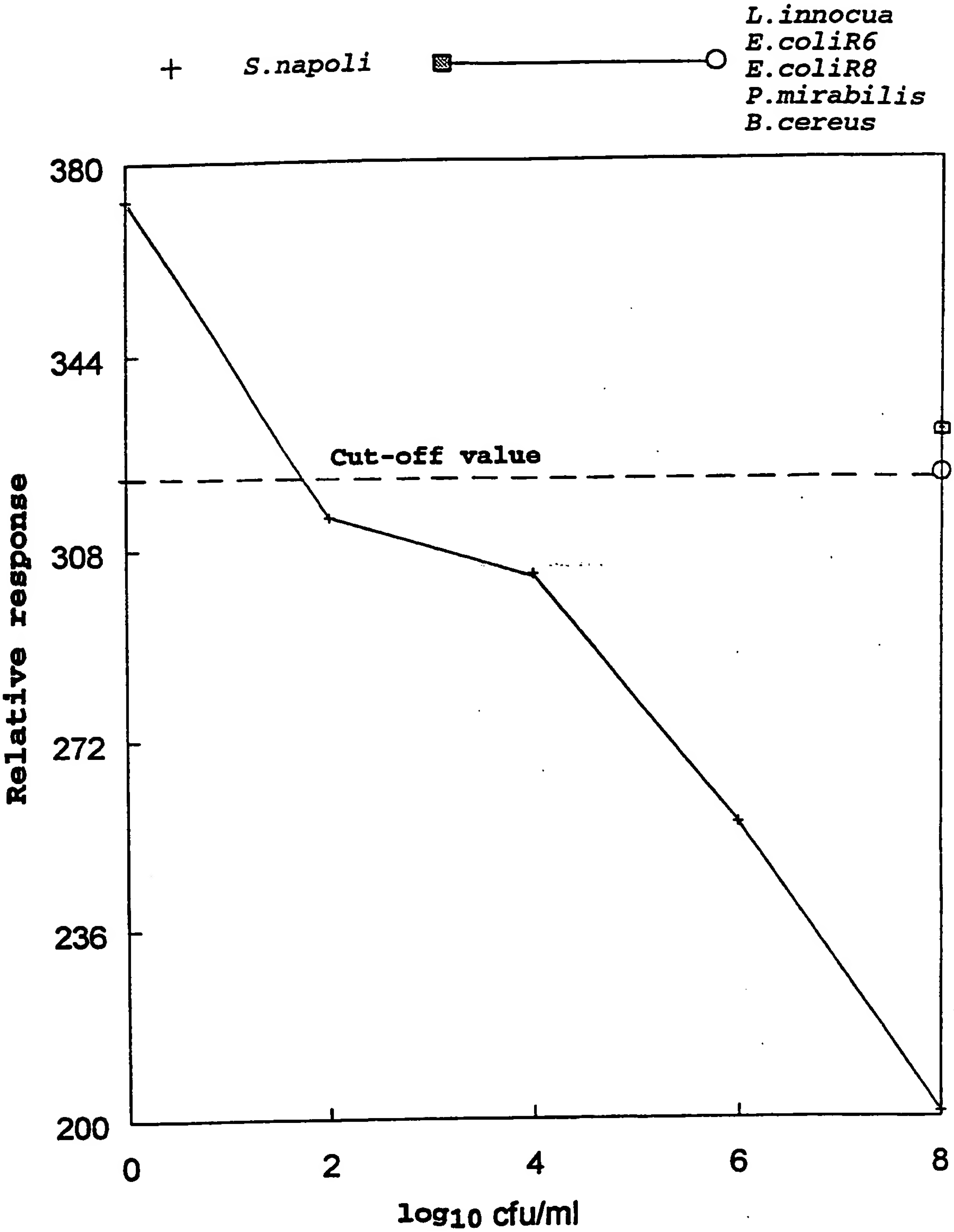
2
4
6
8
log10 cfu/ml

FIG. 2


+ S.napoli
L.innocua
E.coliR6
E.coliR8
P.mirabilis
B.cereus
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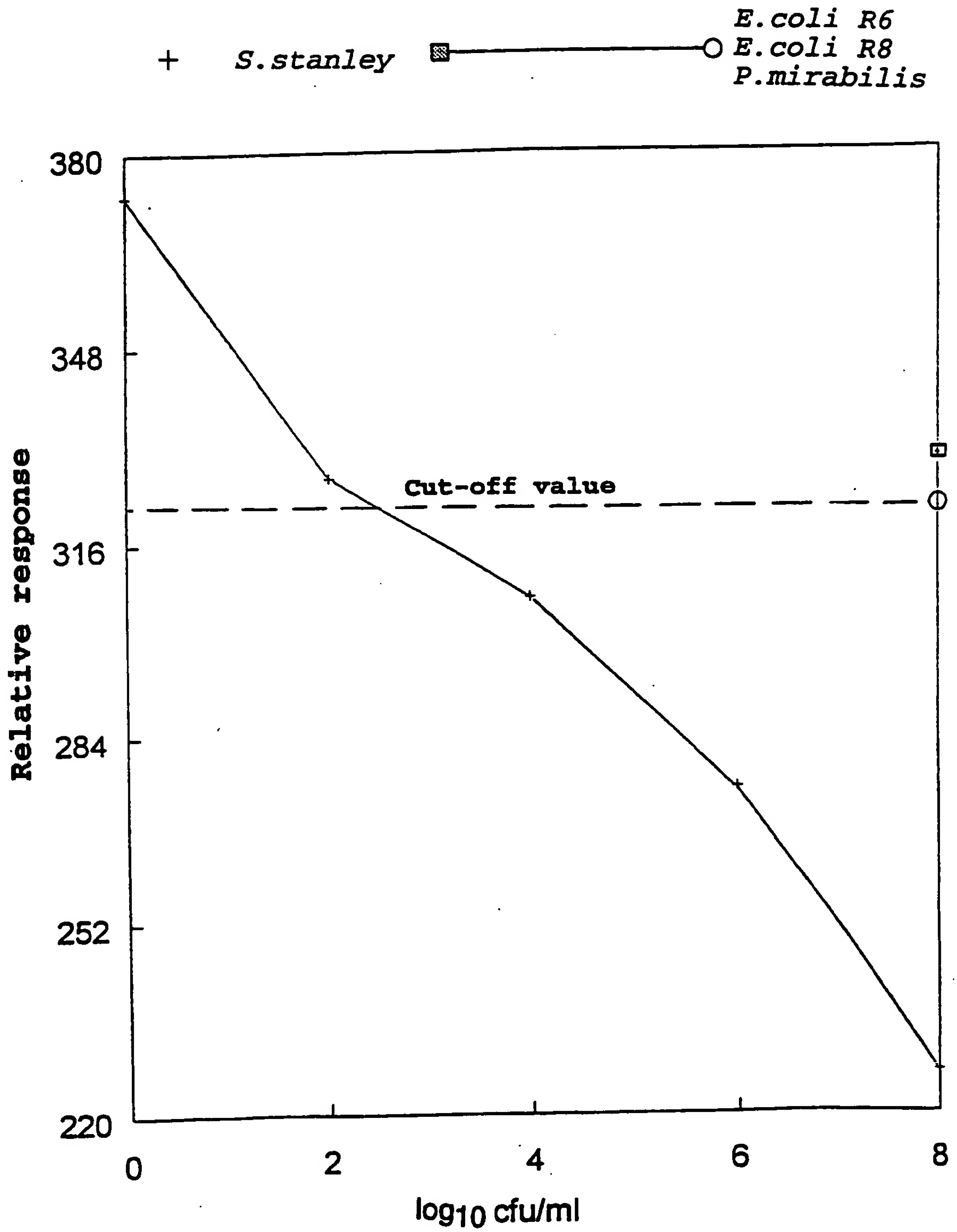
308
272
236
200
Cut-off value
Relative response
0
2
4
6
8
log10 cfu/ml

FIG. 3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E.coli R8
P.mirabilis
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284
252
220
Relative response
Cut-off value
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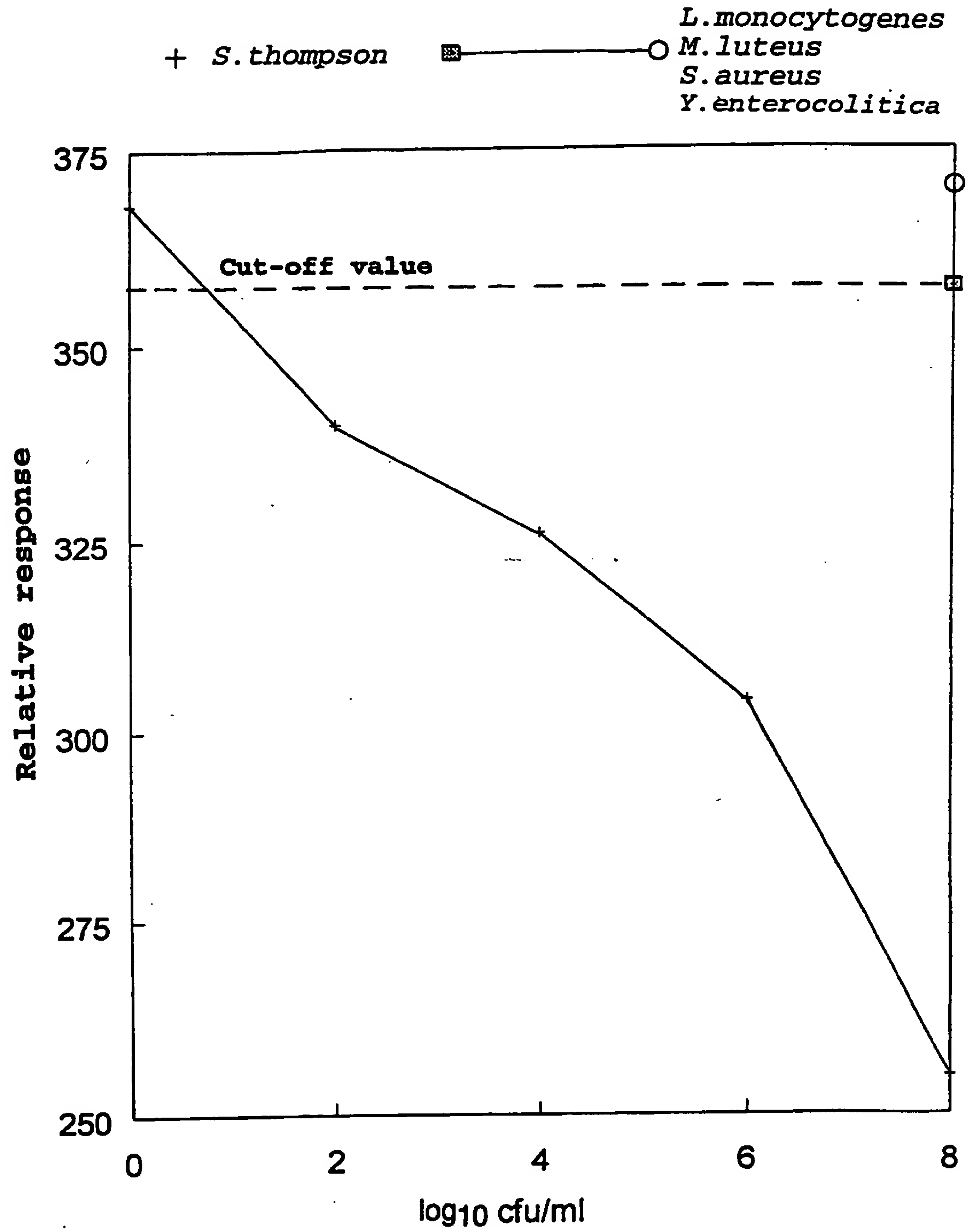
2
4
6
8
log10 cfu/ml

FIG. 4


+ S.thompson
L.monocytogenes
M.luteus
S.aureus
Y.enterocolitica
Cut-off value
Relative response
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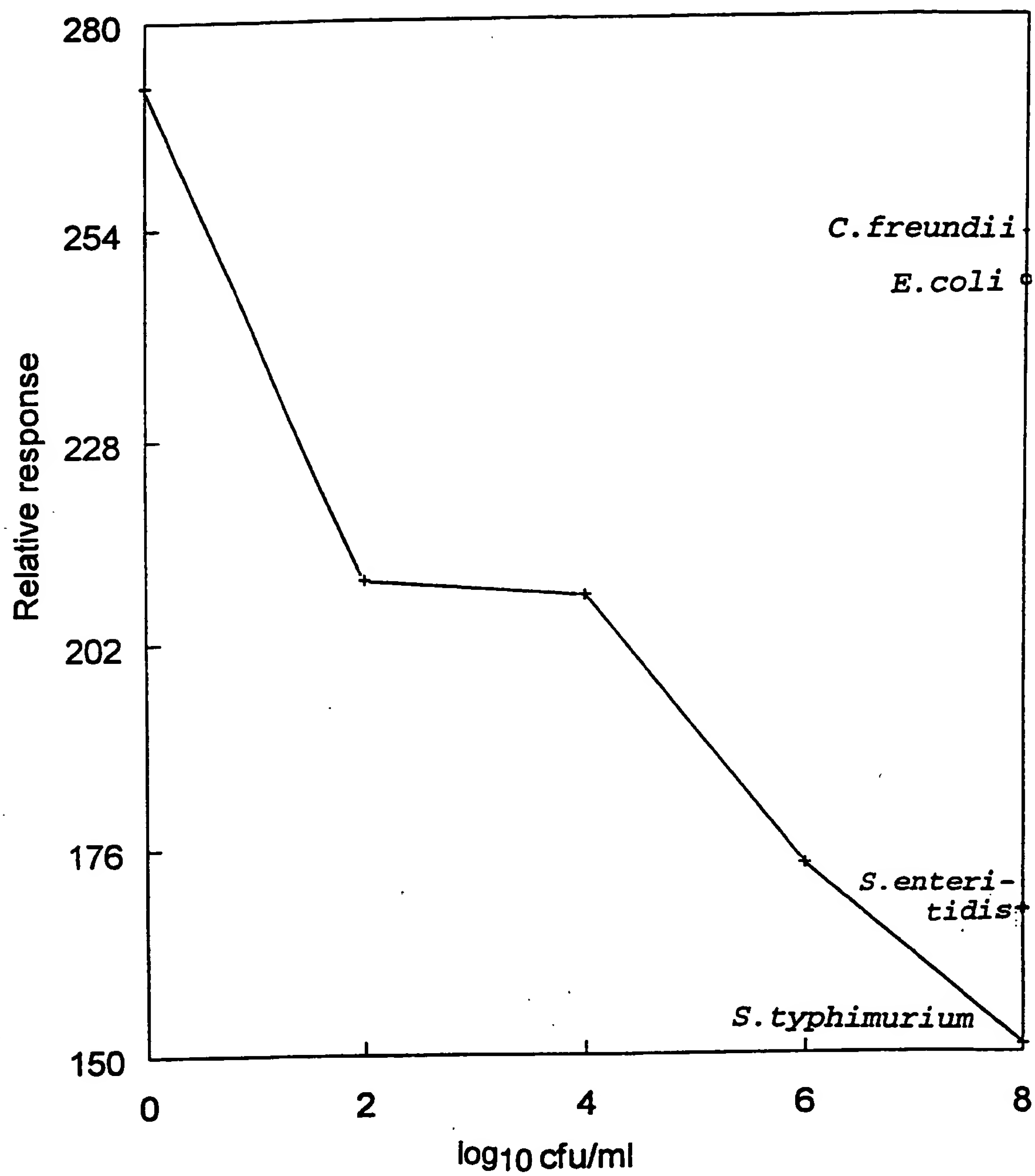
350
325
300
275
250
0
2
4
6
8
log10 cfu/ml

FIG. 5



280
254
228
202
176
150
Relative response
0
2
4
6
8
log10 cfu/ml
C.freundii
E.coli
S.enteri-tidis
S.typhimurium

FIG. 6

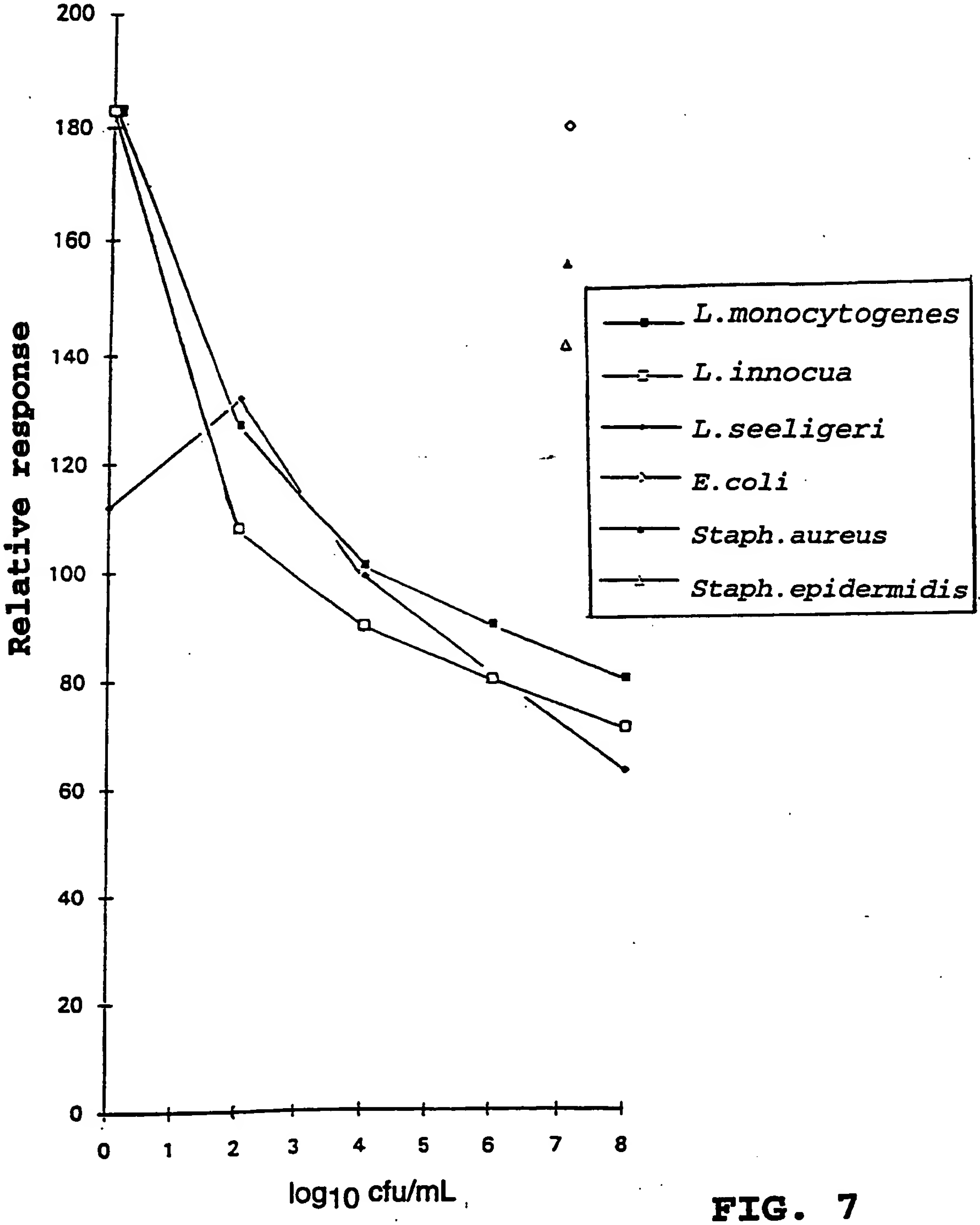
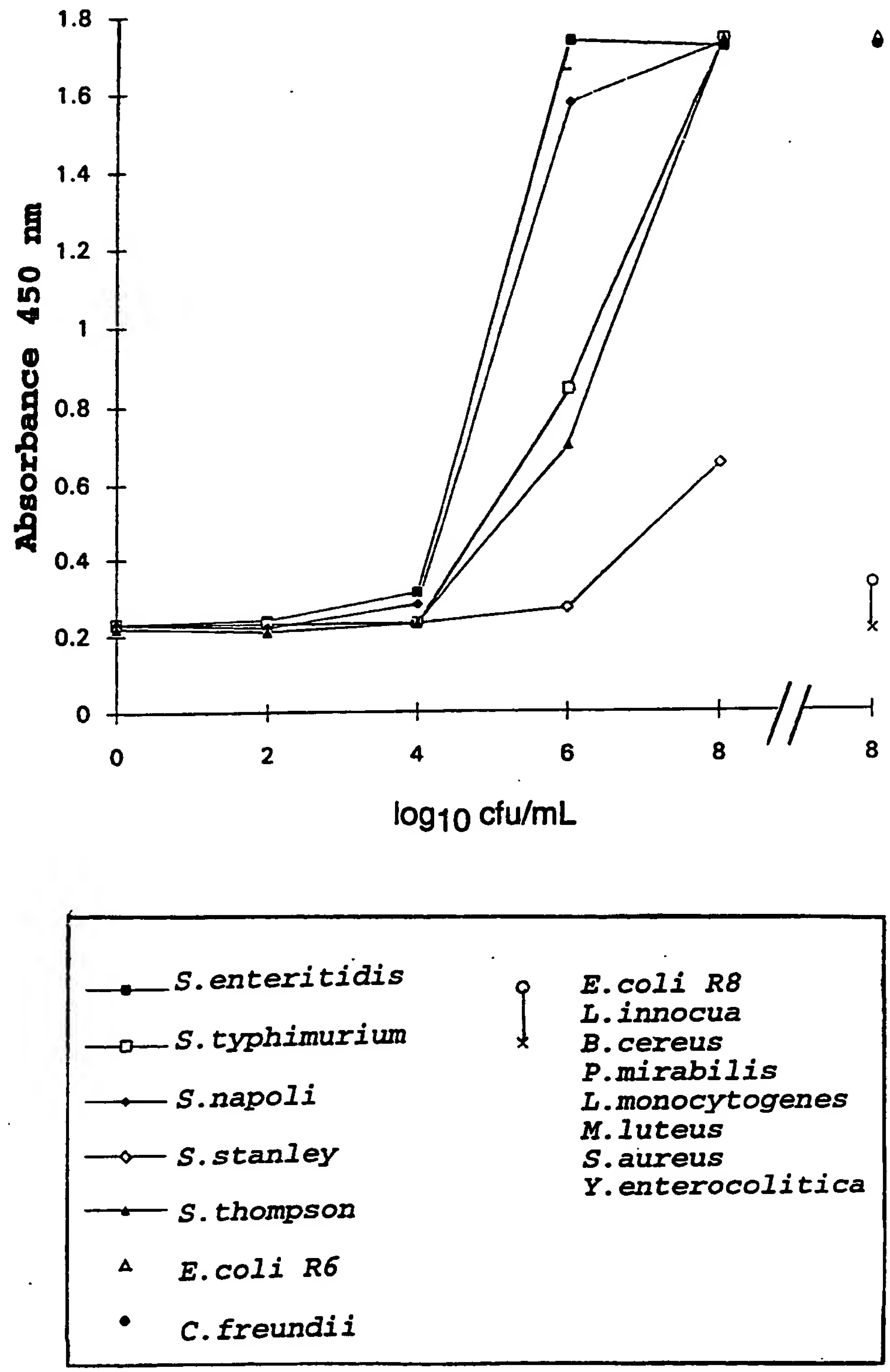

200
180
160
140
120
100
80
60
40
20
0
Relative response
0
1
2
3
4
5
6
7
8
log10 cfu/mL
L.monocytogenes
L.innocua
L.seeligeri
E.coli
Staph.aureus
Staph.epidermidis

FIG. 7



1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance 450 nm
0
2
4
6
8
8
log10 cfu/mL
S.enteritidis
S.typhimurium
S.napoli
S.stanley
S.thompson
E.coli R6
C.freundii
E.coli R8
L.innocua
B.cereus
P.mirabilis
L.monocytogenes
M.luteus
S.aureus
Y.enterocolitica

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 96/00721

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/569, C12Q 1/04, G01N 33/543
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, DBA, WPI, EDOC, PAJ, SCISEARCH, PATENT CITATION INDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Food Protection, Volume 53, No 10, October 1990, Céline Morissette et al, "Simple and Rapid Inhibition Enzyme Immunoassay for the Detection of Staphylococcal Enterotoxin B in Foods" page 834 - page 840	1-2,4,6
A		3,5,7-11
	--	
A	International Journal of Food Microbiology, Volume 12, 1991, S. Notermans et al, "Immunological methods for detection of foodborne pathogens and their toxins" page 91 - page 102	1-11
	--	
A	EP 0496345 A1 (NAGASE & COMPANY, LTD.), 29 July 1992 (29.07.92)	1-11
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Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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International application No.
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